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Nitric Oxide-Mediated Antiplasmodial Activity in Human and Murine Hepatocytes Induced by Gamma Interferon and the Parasite Itself: Enhancement by Exogenous Tetrahydrobiopterin

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Expression of inducible nitric oxide (NO) synthase has been shown to inhibit the development of several pathogens, including fungi, bacteria, parasites, and viruses. However, there is still controversy as to whether this effector mechanism can inhibit the development of human pathogens. We now report that gamma interferon (IFN-γ) induces the elimination of *Plasmodium falciparum*-infected primary human hepatocytes from cultures and that the antimalarial activity is dependent on NO. Infection with the parasite alone in the absence of added IFN-γ caused a 10-fold increase in NO formation. Both spontaneous inhibition and IFN-γ-induced inhibition of *Plasmodium yoelii*-infected murine hepatocytes were increased with the addition of the NO synthase cofactor tetrahydrobiopterin, or sepiapterin, which is converted to tetrahydrobiopterin. These results indicate that under in vitro conditions the parasite itself provides a signal that triggers induction of the NO pathway in human and murine hepatocytes and that NO formation in infected hepatocytes is limited by tetrahydrobiopterin availability.

Numerous cell types, including activated murine macrophages and rodent and human hepatocytes, have been shown to express an inducible nitric oxide synthase (iNOS), which converts L-arginine via an oxidative pathway to citrulline and nitric oxide NO. Activation of this pathway is cytotoxic to some tumor cells and a variety of pathogens (15, 16, 20). Recent evidence indicates that NO is an important effector molecule against the hepatic stages of malaria. By using an inhibitor of NO synthesis, it has been possible to demonstrate that NO accounts for much of the antimalarial activity in murine malaria in vitro and in vivo (10, 12, 18, 22, 25, 26). NO can be induced in vivo in hepatocytes by *Corynebacterium parvum* treatment (2); thus, it is likely that induction of NO accounts for the antiplasmodial activity of this adjuvant seen in vivo (13). Although all stages of the life cycle of *Plasmodium* spp. have been shown to be susceptible to NO, there is still controversy as to whether *Plasmodium falciparum* can be inhibited and/or killed via the NO pathway in human cells (14). Rockett et al. (25) have shown that erythrocytic forms of *P. falciparum* can be killed by exogenous administration of NO donors. We have shown that human hepatocytes express high levels of iNOS when exposed to lipopolysaccharide (LPS), tumor necrosis factor, interleukin-1, and gamma interferon (IFN-γ) (17) and have cloned the cDNA (6) and gene (3) for the human iNOS. What control the parasite itself has on iNOS expression is not known.

NOS activity is dependent not only on the substrate, L-arginine, but also on the availability of reduced cofactors, such as tetrahydrobiopterin (BH_4) (27). Recent data indicate a role for BH_4 in maintaining the NOS enzyme in an active dimeric configuration (1). In many cells that produce NO, including hepatocytes, BH_4 synthesis can be coinduced with NO formation. De novo synthetic as well as salvage and recycling pathways are involved in maintaining BH_4 levels in cells. Thus, we hypothesized that increased NO production by supplementation of cultures with BH_4 would increase inhibition of hepatic stages of malaria development.

The present study was undertaken to address two questions. First, is the human parasite *P. falciparum* inhibited in human hepatocytes by NO; and second, is NO-dependent antimalarial activity limited by BH_4 availability?

MATERIALS AND METHODS

Chemicals, cytokines, and culture reagents. Recombinant human IFN-γ and murine IFN-γ were purchased from Genzyme (Boston, Mass.), and 2,4-diamino-6-hydroxypyrimidine (DAHP) was purchased from R & D Systems (Minneapolis, Minn.). Sepiapterin and 5,6,7,8-BH_4 were from Schirks Laboratories (Jena, Switzerland). N^G-Monomethyl-L-arginine (NMA) was synthesized by the method of Corbin and Reporter (4). Minimal essential medium, insulin, penicillin, streptomycin, L-glutamine, fetal calf serum, and L-arginine hydrochloride were purchased from Gibco (Grand Island, N.Y.).

Sporozoites. Sporozoites were harvested from salivary glands of *Anopheles stephensi* mosquitoes infected with *P. falciparum* (NF 54) or *P. yoelii* (17 XNL, clone 1.1).

Culture of hepatic stages of malaria. Human and murine (BALB/c female mice 6 to 8 weeks old; Jackson Laboratories, Bar Harbor, Maine) hepatocytes were isolated by collagenase (Boehringer, Ingelheim, Germany) perfusion of liver biopsies, as previously described (11, 17). Cells were cultured in eight-chamber plastic Lab-Tek slides (Nunc, Naperville, Ill.) at 10^5 hepatocytes per well for 24 h in 5% CO_2–95% O_2 at 37°C before sporozoite (4×10^4) inoculation. Schizonts were detected in murine and human hepatocytes 2 and 4 days, respectively, after sporozoite inoculation by immunofluorescence assay by a monoclonal antibody directed against a

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specific *P. yoelii* liver-stage antigen (NYLS3) (undiluted supernatant) or a monoclonal antibody directed against *P. falciparum* CS protein (NFS1) (immunoglobulin G concentration, 50 μg/ml). Both antibodies were a gift from Yupin Charoenvit (Malaria Program, Naval Medical Research Program). Percent parasite inhibition was calculated by comparing the number of schizonts in the experimental cultures with that in controls (= 0% inhibition) (10, 11, 18, 19, 21–24).

Induction of iNOS in murine and human hepatocytes. Several cytokines have been shown to participate in the induction of iNOS in rodent and human hepatocytes (6, 7, 17, 21) and affect the development of hepatic stages of malaria via the NO pathway (10, 18, 22). Murine hepatocytes were incubated with 1,000 U of recombinant murine IFN-γ per ml, whereas human hepatocytes were incubated with 1, 10, and 100 U of recombinant human IFN-γ per ml, as described previously (10, 11). The NO-dependent inhibition of pre-erythrocytic stages was demonstrated by using 0.5 mM NMA and an excess of L-arginine (1 and 4 mM).

Increased inhibition of NO-dependent hepatic stages of malaria by BH_4 and sepiapterin. Infected hepatocytes were incubated with 50 μM BH_4 or 75 μM sepiapterin with or without IFN-γ. To assess the involvement of the BH_4 de novo pathway in IFN-γ-induced parasite killing, an inhibitor of BH_4 biosynthesis (1 mM DAHP) was used.

NO_2^- plus NO_3^- measurement in culture supernatants. Culture supernatants were tested 24 and 48 h after sporozoite inoculation for accumulation of NO_2^- plus NO_3^- by an automated procedure based on the Griess reaction, described in detail previously (21).

RESULTS AND DISCUSSION

The capacity of cultured hepatocytes to prevent the development of *P. falciparum* was tested in the presence and absence of IFN-γ. Approximately 40 to 50% of the IFN-γ-stimulated suppression of parasite development was partially prevented by 0.5 mM NMA (Fig. 1A), suggesting that other mechanisms in addition to NO are responsible for the inhibition of *P. falciparum* liver-stage development. NMA added in the absence of IFN-γ increased parasite development by 18%, suggesting a role for NO-induced suppression in the absence of an exogenous costimulus of the NO pathway (Fig. 1B). To show that the effect of NMA was due to the competitive interference of L-arginine conversion, we added excess L-arginine with the NMA to hepatocytes exposed to IFN-γ. L-Arginine at 1 mM partially reversed the NMA effect, which was further substantiated by adding 4 mM L-arginine (Fig. 1B). Other mechanisms that may participate in the suppression of *Plasmodium* spp. development include a mechanism such as induction of reactive oxygen intermediates or acute-phase proteins (19, 23, 24).

Supernatant NO_2^--plus-NO_3^- levels, the stable end products of NO formation, were measured to assess the quantity of NO produced in infected and noninfected human hepatocytes (Table 1). IFN-γ in the absence of *P. falciparum* had no measurable effect on hepatocyte NO synthesis. Inoculation of *P. falciparum* sporozoites in 10^5 hepatocytes alone resulted in the accumulation of 30 nmol of NO_2^- plus NO_3^- over 24 h, a 10-fold increase over that in noninfected cells. IFN-γ and *P. falciparum* acted in synergy to increase NO synthesis by 33% over *P. falciparum* alone. This was increased further by L-arginine supplementation and inhibited by NMA in a manner that was partially reversible by high arginine. These data show increased NO synthesis stimulated by infection alone and synergy between the parasite and IFN-γ. How this occurs is not known, but it does indicate that nonbacterial microbes can stimulate NO synthesis in hepatocytes. NOS activity requires adequate BH_4. We have shown that exogenous BH_4 increases NO formation in cytokine–LPS-stimulated hepatocytes (5). Experiments were performed to determine if the capacity of malaria-infected hepatocytes to inhibit parasite development was limited by BH_4 availability. Due to limited availability of human hepatocytes and previous data showing similar inhibition of *P. yoelii* liver stage in murine hepatocytes, these experiments were performed with this rodent in vitro model (Fig. 2). The spontaneous inhibition of schizont development was enhanced by the addition of BH_4 or sepiapterin, which

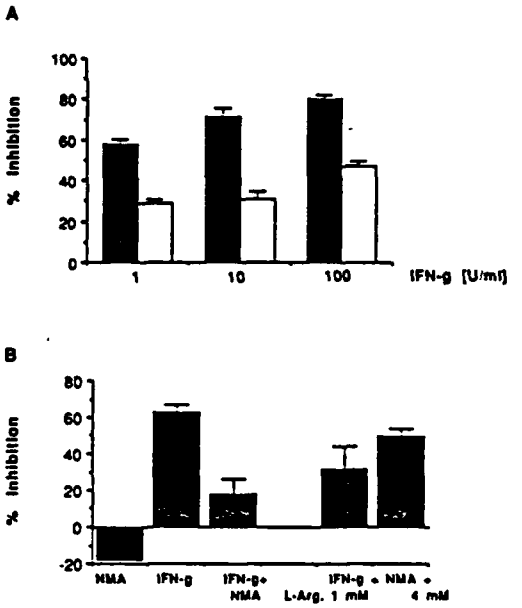

FIG. 1. Inhibition of intrahepatic *P. falciparum* schizonts. (A) Induction of the NO pathway in *P. falciparum*-infected human hepatocytes by IFN-γ. (B) The second set proves the direct NO-dependent inhibition of intrahepatic *P. falciparum* forms. The parasite inhibition in hepatocytes mediated by IFN-γ (10 U/ml) (■) was partially blocked by 0.5 mM NMA (□) (A and B). The NMA effect was overcome by adding excess L-arginine (1 and 4 mM). Basic medium contained 0.25 mM L-arginine. Results are expressed as the mean percent inhibition ± standard error of three independent experiments done in triplicate (control cultures, 53 ± 5 schizonts per well = 0% inhibition).

TABLE 1. NO production in *P. falciparum*-infected and uninfected primary human hepatocytes

Effect of:	NO production (nmol 24 h 10^5 hepatocytes)[a]	
	Uninfected hepatocytes	Infected hepatocytes
Medium[b]		30 ± 1.4
IFN-γ[c]	0.4 ± 0.1	40 ± 1.4
IFN-γ + 1 mM L-arginine	0.4 ± 0.1	53 ± 2.3
IFN-γ + 4 mM L-arginine	0.5 ± 0.1	56 ± 3.6
NMA, 0.5 mM	0.2 ± 0.1	0.2 ± 0.1
IFN-γ + 0.5 mM NMA	0.2 ± 0.1	0.3 ± 0.1
IFN-γ + NMA + 4 mM L-arginine	0.4 ± 0.1	21.9 ± 0.2

[a] NO_2^- plus NO_3^-. Values represent the means ± standard errors for three independent experiments of triplicate cultures.
[b] L-Arginine concentration, 0.25 mM.
[c] 10 U of recombinant human IFN-γ per ml.

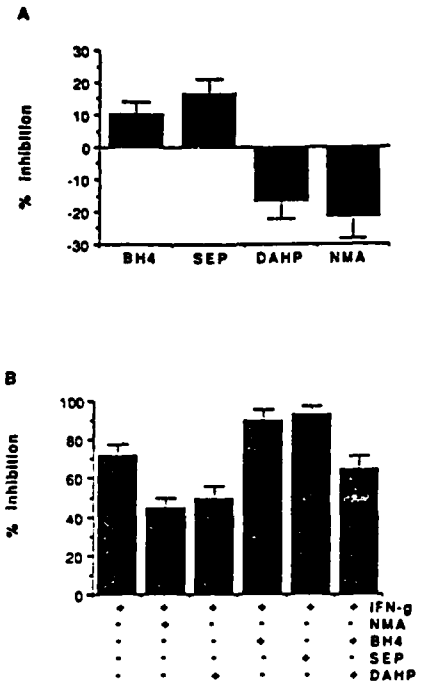

FIG. 2. Enhanced NO-mediated inhibition of *P. yoelii* schizont development by BH_4 and sepiapterin. (A) BH_4 (50 μM) and sepiapterin (75 μM) enhanced the inhibition of *P. yoelii* parasite development, while NMA (0.5 mM) and DAHP (1 mM) increased the development of intrahepatic *P. yoelii* forms. (B) In the second set of experiments, IFN-γ (1,000 U/ml) stimulated *P. yoelii*-infected hepatocytes were incubated simultaneously with a combination of DAHP (1 mM), NMA (0.5 mM), or BH_4 (50 μM). Results are expressed as the mean percent inhibition ± standard error of three independent experiments done in triplicate (control cultures, 168 ± 12 schizonts per well = 0% inhibition).

provides BH_4 by a salvage pathway (8). The addition of DAHP, an inhibitor of BH_4 de novo synthesis (8), suppressed the spontaneous antimalarial activity to a similar degree as the NOS inhibitor, NMA. As with the human cells, a significant increase in inhibition was seen with the addition of IFN-γ. A similar degree of augmentation was seen with BH_4 or sepiapterin addition and a depression was seen with DAHP addition, as measured without IFN-γ addition. In one experiment done with human hepatocytes infected with *P. falciparum*, we found results similar to those in the system with murine hepatocytes infected with *P. yoelii* (data not shown).

BH_4 is an essential cofactor for NOS activity. Although the exact role of BH_4 in NO formation is unknown, recent data indicate that only catalytic quantities are required (9) and that BH_4 participates in forming active dimeric enzyme from the monomer subunits (1). BH_4 levels are controlled by both de novo synthetic and salvage or recycling pathways. Hepatocytes exhibit constitutive pteridine synthesis to provide BH_4 a cofactor for phenylalanine hydroxylase. Nonetheless, we have shown that iNOS expression in hepatocytes requires BH_4 synthesis (5). Recycling or salvage pathways make a smaller contribution to providing BH_4. The mechanisms by which NO interferes with the parasite development are far from being elucidated. However, previous work suggests that inhibition of iron-containing enzymes of mitochondrial respiration, DNA synthesis, and total protein synthesis in the host cell itself (15, 16, 20) and most certainly within the microbial pathogen may play a significant role in NO-induced antimalarial activity.

We show here that both the spontaneous inhibition of the hepatic stage of malaria development and the IFN-γ-induced inhibition can be significantly increased by providing cells with additional BH_4. These data suggest that NO synthesis in *P. yoelii*-infected hepatocytes may be limited by BH_4 availability. Whether this is due to a failure to increase BH_4 de novo synthesis or BH_4 oxidation or consumption by the parasite is not clear. It does suggest that biopterin supplementation may be useful in patients infected with malaria to reduce development. This, however, requires additional investigation.

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